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PROTOCOLS FOR MEASURING MIXED FUNCTION OXYGENASES OF FISH LIVER

P.V. Hodson, P.J. Kloepper-Sams, K.R. Munkittrick, W.L. Lockhart, D.A. Metner, P.L. Luxon, I.R. Smith, M.M. Gagnon, M. Servos and J.F. Payne

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ABSTRACT

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This document was prepared in response to requirements for protocois under Environmental Effects Monitoring (EEM) Programs proposed as part of revisions to Canada's Pulp and Paper Effluent Regulations of the Federal Fisheries Act (1991). Field research programs in Scandinavia and North America consistently demonstrated that activity of liver mixed function oxygenase (MFO) enzymes of fish reflected exposure to chemical inducers associated with effluents from pulp mills using chlorine bleaching. Induction of activity was strongly correlated to other important effects on fish and fish populations. Since the measurement of MFO activity was a logical addition to EEM programs, a workshop sponsored by the Department of Fisheries and Oceans and by Environment Canada was convened at the Freshwater Institute, Winnipeg, Manitoba, in November, 1990 to review the theoretical and practical aspects of MFO measurements. The participants agreed that measurements of MFO activity were suitable and useful indicators of chemical contamination from pulp mills using chlorine bleaching, and that a practical protocol could be prepared. This protocol is the direct result of that workshop and is intended for use by biologists with laboratory experience in biochemistry. This protocol should not be used in isolation, but should be an integral part of EEM programs, as an early step in tier-testing to direct further studies of chemical contamination and biological impacts.

RÉSUMÉ

Hodson, P.V., P.J. Kloepper-Sams, K.R. Munkittrick, W.L. Lockhart, D.A. Metner, L. Luxon, I.R. Smith, M.M. Gagnon, M. Servos and J.F. Payne. 1991. Protocols for measuring mixed function oxygenases of fish liver. Can. Tech. Rep. Fish. Aquat. Sci. 1829: 49 p.

Ce document a été préparé pour le Programme de Surveillance des Effets sur l'Environnement des rejets d'usines de pâte et papier. Ce programme a été entrepris lors du processus de révision des Règlements sur les Effluents de Pâte et Papier de la Loi Fédérale sur les Pêches (1991). Différents programmes de recherche, en Scandinavie et en Amérique du Nord, ont démontré fréquemment et sans exception une augmentation de l'activité des enzymes hépatiques d'oxydase de fonction multiple (OFM) chez les poissons exposés aux effluents d'usine de pâte et papier avec blanchiment au chlore. Cette induction de l'activité enzymatique est fortement associée à d'autres effets importants chez les poissons et les populations de poissons. Comme la mesure de l'activité des OFMs est une composante logique d'un programme de surveillance des rejets de pâte et papier, le Ministère des Pêches et des Océans et Environnement Canada ont organisé un atelier ayant comme objectif d'examiner de façon critique les aspects théoriques et pratiques des mesures d'activité des OFMs. Cet atelier a eu lieu à l'Institut des Eaux Douces, à Winnipeg, en Novembre 1990. Les participants ont reconnu l'utilité et la pertinence des mesures de l'activité des OFMs comme indicateurs de la contamination par des effluents de pâte et papier avec blanchiment au chlore et ont proposé de préparer un protocole décrivant les méthodes de mesure des OFMs. Ce protocole est donc un résultat direct de cet atelier. Il a été conçu pour être utilisé par des biologistes avec de l'expérience de laboratoire en biochimie. Ce protocole ne devrait pas être utilisé comme unique méthode de surveillance mais devrait constituer une étape préliminaire au sein d'un programme complet de surveillance de la contamination chimique et des impacts biologiques.

1.0 INTRODUCTION

Monitoring the effects of industrial wastes in receiving waters includes monitoring the responses of fish. Fish are present in most Canadian surface waters and their welfare is relevant to the public, to environmental control, and to sports and commercial fisheries. The measurement of the activity of fish liver detoxication enzymes, the mixed function oxygenases (MFOs), has been proposed as a tool for monitoring the environmental effects of marine petroleum pollution (Addison and Payne 1986). MFO activity has been tested as an indicator of the presence of chemical contamination from urban and industrial development in over 30 field trials (Payne *et al.* 1987), and has been included in the North Sea Monitoring Master Plan (Addison 1991). This document describes the measurement of fish liver MFO activity as a tool for monitoring chemical contamination associated with effluent from pulp mills using chlorine bleaching (Bleached Kraft Mill Effluent or BKME). It includes the biochemistry, toxicology and measurement of the MFO enzymes in fish and it identifies what data should be collected, how they should be collected, and how positive and negative responses should be interpreted.

1.1 Theory

Mixed function oxygenases (also called mixed function oxidases, MFOs, cytochrome P-450s, polysubstrate multifunction oxygenases, PSMCs) are a family of membrane-bound enzymes which increase the water solubility of aromatic and lipophilic compounds (White and Coon 1980). Natural substrates for MFOs include endogenous compounds such as steroid hormones and fatty acids, although they also aid in drug metabolism and the breakdown and excretion of other exogenous compounds (Lu and West 1980; Ryan *et al.* 1979). MFO activity includes various reactions which add molecular oxygen to lipophilic compounds. The terminal oxidase enzyme of the MFO system is the iron-containing hemoprotein cytochrome P-450 (Blumberg 1978); the '450' refers to the wavelength absorption maximum of the reduced form of the cytochrome after complexation with carbon monoxide. One group of cytochrome P-450s, called P-450IA, is 'induced' by the presence of several foreign compounds. That is, in the presence of these foreign compounds, animals synthesize new amounts of P-450IA proteins and enzyme activity is measurably increased.

Induction is initiated when a foreign compound binds to a specific cellular receptor (Poland *et al.* 1976; Perdew and Poland 1988), often called the Aryl hydrocarbon or Ah receptor. Binding triggers the expression of the gene coding for P-450IA leading to increased RNA transcription (Gonzalez *et al.* 1984; Israel and Whitlock 1984) and eventual synthesis of new P-450IA protein. Induction can be detected by measures of several components in the chain of cellular events: P-450IA mRNA, P-450IA protein, or P-450IA enzymatic activity (Nebert and Gonzalez 1987).

While most studies of P-450IA originally concerned mammals, the system is also inducible in fish. Experimental treatments with pure compounds have established that some polynuclear aromatic hydrocarbons (PAH: benzo(a)pyrene, 3-methylcholanthrene) and some chlorinated

aromatic hydrocarbons (co-planar PCBs, chlorinated dibenzodioxins and dibenzofurans) induce liver P-450IA in several species (James *et al.* 1979; James and Bend 1980; Förlin 1980; Gooch *et al.* 1989; Law and Addison 1981; Vodicnik *et al.* 1981; Addison *et al.* 1982; Van der Weiden *et al.* 1990a; 1990b; Muir *et al.* 1990). The ability to induce MFO activity appears to be related to molecular shape, i.e. the co-planarity of connected aromatic rings and the distribution of substituents such as chlorine atoms. Complex mixtures such as Aroclors, petroleum oils, and BKME also have inducing properties (Förlin and Lidman 1981; Melancon *et al.* 1981; Addison *et al.* 1982; Melancon and Lech 1983; Payne *et al.* 1988; Walton *et al.* 1978; Walton *et al.* 1983; Andersson *et al.* 1988; Thomas *et al.* 1989), probably because these mixtures contain specific inducers.

1.2 Interpretation

Induction of P-450 enzymes reliably indicates exposure to compounds with inducing properties. Field studies of P-450IA in fish have focused on oil spills, PAH contamination of sediments and, more recently, BKME (Payne 1976; Elskus and Stegeman 1989; Dunn 1980; Johnson *et al.* 1988; James and Bend 1980; Van Veld *et al.* 1990; Andersson *et al.* 1988; Rogers *et al.* 1989). Scandinavian and Canadian studies of fish captured near sources of BKME have shown dramatic induction of P-450IA-related enzymatic activities when compared with fish from reference sites (Andersson *et al.* 1988; Lindström-Seppä and Oikari 1989; Rogers *et al.* 1989, Sodergren 1989; Munkittrick *et al.* 1991a; Smith *et al.* 1991; Hodson *et al.* 1991; Servos *et al.* 1991). Studies in Canada were of effluents from five bleached kraft mills discharging to Lake Superior, Thunder Bay (Lake Superior), the Athabasca River, the Spanish River and the St. Maurice River. Since PAHs and PCBs are not commonly found in pulp mill effluents, MFO induction may indicate exposure to dioxins and furans, and both have been measured in effluents and the tissues of induced fish (Rogers *et al.* 1989; Hodson *et al.* 1991).

Recent experiments suggest that other compounds in bleached and unbleached mill effluents may also be responsible for induction, but the evidence so far is circumstantial and the identities of the inducing agents are as yet unknown. For example, Munkittrick *et al.* (1991b) observed that inducton in fish caught near a bleached kraft mill disappeared when the plant temporarily ceased operations, suggesting the discharge of an inducer that was not persistent, in contrast to dioxins and furans. Payne and Fancey (1981) observed low levels of induction in both liver and gill of rainbow trout exposed to shavings of wood bark floating in aquaria and they speculated that some turpenoid hydrocarbons may be inducers.

Studies of mills not using chlorine bleaching are lacking in Canada and results of such studies in Scandinavia are conflicting. Swedish studies of mills not using chlorine bleaching indicate no induction (Larsson *et al.* 1988), whereas a recent Finnish study showed the opposite (Lindström-Seppä *et al.* 1991). Since dioxin discharges from pulp mills have been associated in the past with the use of chlorophenolic-treated wood chips, the presence of dioxins in these studies cannot be discounted without analyses of both fish and effluents, and the question of other inducing agents remains open. Nevertheless, detection of MFO induction in fish from areas

contaminated by BKME is a strong argument for surveys of chemicals typical of BKME exposure, in particular for dioxins and furans in fish tissues. Chemical surveys of sediment and fish tissue will help to determine whether the pulp mill is the source of contamination and whether induction was due to the presence of PAHs from combustion or to PCBs from electrical equipment.

The biological significance of P-450IA induction is not completely known. Induction is an adaptive response and can result in the metabolism and excretion of exogenous substrates. Studies demonstrating increases in P-450IA activity have also documented changes in fish performance, including altered steroid hormone profiles, changes in thyroxine and vitamin A metabolism, impairment of the reproductive and immune system, and an increased prevalence of diseases (e.g. Munkittrick *et al.* 1991a; Larsson *et al.* 1988). There has not yet been a demonstration of causal links between altered P-450IA activity and other biochemical responses of fish (e.g. altered hormone profiles); one hypothesis is that both MFO induction and hormone effects are the result of binding by the contaminants to the Ah or other receptors, but are not otherwise related.

The simultaneous occurrance of adverse effects and MFO induction indicates that measures of MFO induction justify further studies of biological impacts. The area in which P-450IA activity is altered is assumed to represent the zone of direct influence of the effluent on fish physiology and performance. The philosophy behind using P-450IA for monitoring populations subject to BKME is one of simple prudence. Induction is one of the easiest and most sensitive responses to detect. If induction can be avoided, presumably other Ah-associated biological responses will also be avoided. If induction is detected, more detailed studies are needed of the survival, growth, reproduction, and bioaccumulation of inducers by the local fish community. It must be recognized, however, that lack of induction does not mean 'no effect' - other effects may be produced by biochemical actions independent of the Ah receptor. Measurement of MFO induction signals only an increased probability of a suite of associated responses.

Induction without measureable levels of chlorinated compounds ('false positive') is also possible if fish have been exposed to PAHs from other industries. In this case, the enzyme activity will reduce levels of PAHs in fish tissues to 'non-detectable' and chemical analysis of sediments will be required to confirm that contamination does not come from the pulp mill, particularly in areas where many industries discharge to the same water body.

In summary, the P-450IA enzyme system is a consistent, simple and economical indicator of exposure to inducing contaminants; its response in fish signals the need for surveys of chemical contamination and more detailed biological studies of effects on fish.

1.3 Caveats

The induction of MFO activity represents a chain of cellular events that includes binding of the inducing compound to the Ah receptor, production of messenger RNA to initiate synthesis of new enzyme proteins, and the action of the enzymes on various substrates. Each step of the chain can now be measured, with varying degrees of difficulty. Currently, the simplest is the measurement of enzyme activity, as described in the appendices to this protocol. However, since this is the last step in the chain, it is the most sensitive to interference; with more steps in a chain, there is a greater possibility of an interference. There are many labs developing methods to measure the quantity of the protein enzyme and of the messenger RNA. Refinement of these techniques could lead to future 'kit' methods that are more sensitive, more reliable, faster and less expensive than the methods described here.

Studies of various species (Table 1) consistently demonstrate that MFO induction reflects chemical exposure. However, species differences in the magnitude of the response are found, and both biological variables (sex, age, spawning status, size, genetic strain) and habitat variables (temperature) may influence the response (Stegeman and Chevion 1980; Koivusaari *et al.* 1981; Luxon *et al.* 1987; Lindström-Seppä 1985; Pedersen *et al.* 1976; Jimenez and Burtis 1989).

The most important biological factor affecting activity is reproduction. While there is little difference between the sexes for immature fish, mature females have slightly lower MFO activities than males, a difference accentuated during spawning when hormones such as estradiol vary dramatically in their concentrations in serum (Förlin and Haux 1985). While differences among treated and control sites can still be seen, increased variability and lower activities may obscure statistical significance. Each species should be sampled during its inter-spawning interval, at least two to six months before spawning, and fish must be carefully selected for a similar size, stage of development, age, sex and feeding activity.

Age and life stage are important co-variates of sexual maturation. They influence the distribution of fish and their diet, and hence their exposure to pulp mill effluent. For example, spawning migrations may control the time spent in the effluent and whether the fish are truly representative of the region in which they are caught. There are also very large species differences in the induction measured during field studies, which may be due to variations in exposure, accumulation or sensitivity to inducers. In a responsive species such as longnose sucker, a five-fold induction is not equivalent to a five-fold induction in species which are less responsive, such as lake whitefish or walleye.

Temperature is an important environmental variable, since the activity of enzyme molecules declines with temperature; less energy is available to drive chemical reactions. Since acclimation or thermal compensation requires time and is species-dependent, temperature may affect both inducibility and MFO activity.

Methods of fish capture, tissue handling and analysis of enzyme activity are potential sources of analytical error, so these factors must be standardized among sites. There appears to be no

Table 1. Species of fish showing MFO induction. This list is restricted to those species reported in papers listed in Section 9.0, References.

	COMMON NAME	LATIN NAME
FRESHWATER		
	Rainbow trout	*Oncorhynchus mykiss*
	Chinook salmon	*Oncorhynchus tshawytscha*
	Lake trout	*Salvelinus namaycush*
	Brook trout	*Salvelinus fontinalis*
	Lake whitefish	*Coregonus clupeaformis*
	Vendace	*Coregonus albinus*
	Carp	*Cyprinus carpio*
	White sucker	*Catostomus commersoni*
	Longnose sucker	*Catostomus catostomus*
	Bluegill	*Lepomis macrochirus*
	European Perch	*Perca fluviatilis*
	Walleye	*Stizostedion vitreum*
BRACKISH		
	Killifish	*Fundulus heteroclitus*
	European Perch	*Perca fluviatilis*
MARINE		
	Scup	*Stenotomus chrysops*
	Cunner	*Tautogolabrus adspersus*
	Winter flounder	*Pseudopleuronectes americanus*
	English sole	*Parophrys vetulus*
	Spot	*Leiostomus xanthurus*

obvious effects of partial suffocation (e.g. by gill netting), electroshocking or capture stress on MFO activity (L.L. Luxon and P.V. Hodson, unpublished data). Prolonged stress may contribute to water retention and enlarged livers, which would bias activity expressed as 'per gram of tissue'. However, measurement of specific activity (i.e. activity per mg of protein in liver extracts) ensures a reliable measurement. An uneven distribution of enzyme activity within livers can contribute to analytical error; either the entire liver must be minced and mixed before analysis to avoid heterogeneity, or subsamples must be taken from the same area of liver in each animal sampled. Other effects due to dissection, tissue storage and assay procedures are alluded to in the detailed methods, and a quality control program is outlined.

The methods presented here have been widely used in research and in pilot monitoring programs. A ring test among ten laboratories using the fluorometric ethoxyresorufin-o-deethylase (EROD)

method (Appendix B) demonstrated that all could separate six induced from six un-induced fish with no overlap or errors, although measures of absolute activities for each fish varied among labs. Therefore, it appears that all labs could identify responses of fish to pulp mill effluent, should they occur (Munkittrick *et al.* 1991c).

In summary, confounding variables such as temperature, fish size, sex, maturity and season must be standardized as much as possible among contaminated and reference sites. Sampling programs should avoid the spawning period of the target species or increase the number of fish sampled. Using these designs in field studies and the specific procedures outlined for each analytical method, the MFO system can successfully detect specific chemical exposures and can indicate whether more costly chemical analyses are needed.

2.0 SAMPLE COLLECTION AND STORAGE

2.1 Study design and choice of fish species

Sampling should include two species, at least one benthivore and one pelagic predator. MFO activity of the benthivore will reflect sediment contamination and MFO activity of the predator will reflect accumulation of inducing compounds through the food chain. Sample size should be based on study design and requirements, but a minimum of 10 to 12 fish per site must be taken and 15 are recommended. Sequential statistical analyses of liver aryl hydrocarbon hydroxylase (AHH) activities of 20 fish showed that running means and standard deviations stabilized only when sample sizes were 10 or more (P.V. Hodson, unpublished data). It is not always possible to collect adequate numbers of both sexes of fish at all field sites, during all seasons. Since there may be differences between sexes for most species, this should be taken into consideration during study design, possibly by restricting sampling to male fish.

Sampling must always include a minimum of one contaminated site and one uncontaminated reference site, but two reference sites are preferable, with one immediately 'upstream' of the source. The reference sites should be chosen to decrease the influence of fish movements and migration. Sampling at two reference sites may not be required if a) fish are prevented by barriers from moving between the upstream and downstream sites, or b) sufficient data are available from other sources to validate the data from one reference site. Where more than one test site is possible, they may be positioned to demonstrate the spread of the effluent in a lake or ocean, or exposure gradients caused by distance or by dilution from tributaries.

There are areas where studies on free-ranging wild fish may not be possible, especially where pulp mills are located adjacent to one another. One solution is to cage fish, with cages distributed among contaminated and reference sites. A number of studies have found that caging fish in pulp mill effluents for periods of up to 30 days does not induce MFO activity to the same extent as in native fish, although induction is often evident (Lindström-Seppä and Oikari 1989). The cause of the difference has not been defined.

2.2 Field sampling procedures

There are several alternatives for sampling: samples may be processed entirely in the field, partially processed in the field and completed in the laboratory, or processed entirely in the laboratory. Although it is possible to conduct the entire assay under field conditions (see Addison and Payne 1986), this is not always necessary or practical. The samples can be stored until transferred to the laboratory, but storage conditions are critical. Freezing and thawing samples cause the loss of 30-40% of the original activity, but the loss of activity is uniform among sites (i.e. that the exposed site will show the same relative induction). Methods for collecting, processing and storing liver samples must be standardized among sites.

Liver must be collected from live fish; MFO activity decreases and variability increases within 15 min of death, depending on external temperature. There are several ways to process samples in the field. Livers must be removed carefully, avoiding the rupture of the gall bladder and contact with bile. Sub-samples taken from one area of the liver or whole livers may be removed intact, and frozen immediately, or rinsed in 0.15 M KCl and minced prior to freezing on dry ice or liquid nitrogen. The advantages of mincing the liver are that the mix represents the average MFO activity throughout the liver, since the activity can vary by < 2 - 3 fold among various parts of the liver. A third alternative is to homogenize directly in the field with a buffer containing glycerol and to freeze the homogenate for lab analysis. EROD activity has been compared between homogenates prepared in the lab and field from portions of the same livers. There was consistently lower activity with field homogenates but the same relative order of activity (M.M. Gagnon, unpublished data; A-M. Prud'homme and J. Bureau, Environment Canada, personal communication; K.R. Munkittrick, unpublished data).

There are reports of MFO inhibition or interference with fluorescence readings and P-450 spectra if traces of bile or blood are present. As a simple precaution after dissection and weighing, the liver must be rinsed immediately with cold 0.15 M KCl to remove traces of blood and bile. For an animal with a simple liver, the severed blood vessels provide a convenient entry for irrigation through the tip of a squeeze bottle. For a multi-lobed liver, the best alternative is external washing. K.R. Munkittrick (unpublished data) found no effects of mincing the liver of white sucker, or of the position within the liver (anterior versus middle or posterior lobes) on EROD activity. However, when the liver was minced prior to freezing, there was a decrease in activity unless the sample was rinsed in KCl.

For tissue storage, all samples must be quick-frozen in liquid Nitrogen or dry ice and stored at -60°C or lower; storage at -20°C is not acceptable, even for a very short period of time. Livers homogenized in the field require more liquid Nitrogen to freeze a given weight of liver due to dilution by buffer. Freezing is most efficient for small portions, e.g. as 2.0 g aliquots in cryovials.

In the laboratory, frozen tissue is thawed, homogenized and centrifuged to separate MFO enzymes from cell debris. The first stage is a low-speed (9000 x g) centrifugation that gives a 'post-mitochondrial supernatant (PMS)', also called S-9 fraction. The second stage is a centrifugation of the S-9 fraction at 100,000 x g, which separates the enzyme in a pellet of microsomes (membrane fragments). Although some authors prefer the use of the microsomal preparation for MFO assays, there is a very strong correlation between MFO activity determined in the S-9 fraction and in the more highly purified microsomes (r^2>0.99). Even though the fluorescence from a microsomal preparation is 3 - 4 x higher than from S-9, the relative amount of induction is similar (K.R. Munkittrick, unpublished data). The lower centrifuge speeds required for S-9 preparations allow production of test material with portable centrifuges. Thus, a fourth alternative is to prepare S-9 fractions in the field from fresh livers, freeze the supernatant in liquid Nitrogen, and analyse enzyme activity in the laboratory. This approach requires more work and equipment in the field but may reduce the loss of activity associated with freezing minced tissues or homogenates.

Microsomal preparations may be frozen at -80°C by resuspension in homogenizing buffer containing 20 % glycerol. Activity remains constant for at least a year and repetitive analyses of multiple frozen aliquots of the same homogenate provides an internal standard as a check on the precision of the chosen method. The amount required per aliquot is the pellet derived from preparing 1 - 2 g of liver.

Details of these procedures are given in Appendix A, Preparation of S-9 fractions and microsomes.

3.0 CHOICE OF MFO ASSAY

There are several methods available for measuring cytochrome P-450IA in fish (details are presented in Appendices B-E). Recent developments include P-450IA protein and mRNA measurement and will be discussed briefly below. The most common methods employ catalytic assays, using substrates that are metabolized in specific ways by the P-450IA enzyme. P-450IA belongs to a gene superfamily (Nebert and Gonzalez 1987) and often shares overlapping substrate specificities with other forms, so substrates other than those described in this protocol must be chosen with care and with knowledge of substrate specificities.

Substrates for P-450IA include benzo(a)pyrene and 2,5-diphenyloxazole (aryl hydrocarbon hydroxylase or AHH assays) and ethoxyresorufin (ethoxyresorufin-o-deethylase or EROD assays). For each substrate, various methods exist, the major ones being radiometric, fluorometric and spectrophotometric. The appendices describe methods used frequently by the authors, although many variants have been published in the literature. For example, the spectrofluorometric EROD assay of Pohl and Fouts (1980) is described, but the version by Lake (1987) gives equivalent results (P. Martel, Pulp and Paper Research Institute of Canada, 570 St. John's Boulevard, Pointe Claire, Québec H9R 3J9, personal communication). Users are encouraged to consult the original references cited for further information on this assay. The choice of assay method depends on each laboratory, considering substrate source (e.g. purity), assay cost, ease and speed of analysis, sensitivity and reliability of the assay. Regardless of the method chosen, certain aspects must be controlled and understood for each species examined to obtain consistent and credible results. These include protein concentration, NADPH concentration, substrate concentration and incubation temperature. Whichever enzymatic analysis is chosen, the limits of detection must be recorded, and the limits will vary among samples according to their protein contents. While detection limits are not as important for high activity samples, they are crucial for samples at or near the limits of detection. As part of quality assurance and quality control (QA/QC), high and low activity "reference" samples must be run to assure consistency in results obtained with unknown samples. QA/QC issues are discussed in section 5.0.

3.1 Other techniques for measuring P-450 expression

Besides enzymatic analysis, other techniques for measuring P-450IA expression include direct protein quantitation and mRNA analysis. Analysis of mRNA is somewhat unwieldy and inappropriate for routine analysis; current technology requires RNA purification and use of a ^{32}P-labelled cDNA probe. However, P-450IA mRNA measurement is essential in situations when enzyme inhibition and/or degradation is suspected at the same time as gene activation (e.g. after high doses of co-planar PCBs). The P-450IA protein measurement is relatively quick and easy in experienced hands. It is inherently more robust than assays of enzyme activity (e.g. it works well even with degraded samples), but requires specific antibodies. An excellent "reagent" antibody exists which is highly specific for P-450IA and which it can detect in mammals, birds, reptiles, and fish (Park *et al.* 1986; Kloepper-Sams *et al.* 1987). Unfortunately, this specific

anitbody is not commercially available, although antibodies against rat P-450IA1 and oligonucleotide probes for rat P-450IA1 RNA are sold by OXYGENE, a company in Dallas, Texas. These reagents have not been tested in studies of MFO induction in fish exposed to pulp mill effluents.

4.0 DATA

4.1 Data requirements - field

MFO measurements are not made in isolation. Aside from associated environmental measurements described in other protocols (e.g. temperature, alkalinity, pH), there are several measurements and samples that must be collected from each fish during the necropsy. These include: total body weight (g), gutted carcass weight (weight (g) after removal of intestines and gonads), gonad weight (g), liver weight (g), fork length (cm), sex, and age (y). In immature fish, sex can be determined histologically from a thin section of gonad preserved in buffered formalin. Age is measured by counting annuli in hard structures such as otoliths and cross-sections of fin rays, cleithra or opercular bones, depending on the species. Use of scales is not recommended for fish greater than 5 y of age.

From these measurements, the condition factor (CF= 100 x (total weight - gonad weight)/length^3), gonadosomatic index (GSI = 100 x (gonad weight/gutted weight)) and liver somatic index (LSI = 100 x liver weight/gutted weight) are calculated. Condition factor is based on gonad-free weight to remove bias due to variations in sexual maturation, and GSI and LSI are based on gutted weight to remove bias due to variable levels of fat in gonads and intestines and variable gonad weight. Correlations should be calculated between enzyme activity and sex, CF, LSI, GSI, weight and age to identify possible bias in MFO results. These factors can help to identify differences in exposure to inducers as a result of sexual maturation and habitat selection by different sizes of fish. Bias would indicate the need for stratified sampling and factorial analyses of variance to compare activity among sites.

Other important observations are unusual coloration or lumps in the liver, or other obvious pathology in other tissues. These measurements are the bare minimum, and if there are associated studies on fish population structure, many more could be added.

Where *post-hoc* confirmation of MFO induction by chemical analyses is possible, samples should be taken of sediments and fish tissue (usually whole carcass and liver) and they should be deep frozen as quickly as possible in contaminant-free plastic bags or aluminum foil rinsed in ultra-pure hexane. Analyses for trichloroguaiacols (ug.kg^{-1}) and chlorinated dioxins and furans (ng.kg^{-1}) would reflect exposure to BKME and the presence of chemical gradients, and would help to confirm that responses were due to pulp mills and not to other industrial effluents.

4.2 Protein content of the liver

Since measurements of MFO activity are an expression of the catalytic activity of protein molecules, it is expressed as 'specific activity', i.e. activity per mg of protein in the microsomal or S-9 preparation. The total protein content of either the microsomal fraction or the S-9

fraction, whichever is used for the MFO determinations, must be measured using established protein assays with bovine serum albumin as a standard.

An option is to measure the amount of P-450 protein (Appendix F) to calculate the 'turnover number', i.e. activity per nmol P-450 protein or the ratio of substrate molecules metabolized per molecule of enzyme. Turnover numbers may be misleading since P-450s are a family of proteins that include many different enzymes. Since some inducers may inhibit P-450IA, induce other P-450 enzymes, or change the protein or lipid content of liver, the turnover number may not reflect induction. However, a comparison of specific activities and turnover numbers among sites may help to identify mechanisms of toxicity. Total cytochrome P-450 content can be measured using a scanning spectrophotometer (Appendix E).

The total capacity for liver MFO metabolism can be calculated by multiplying the specific activity by the mg of microsomal protein per g of liver (protein yield) to give activity per gram of liver. Activity per liver (activity per gram multiplied by the total weight of the liver) can also be calculated, but it is subject to bias caused by differences in fish size among sites, unless fish are selected for a uniform weight or a correction is applied for relative liver weight by normalizing fish weights to a common value.

4.3 Statistics

Assuming that at least five fish have been caught at each site (10-15 is the ideal), the data must be analyzed statistically following the study design. It is a general phenomenon that biological data are not distributed normally, but are skewed towards higher values. All data must be tested for normality and homogeneity of variance before applying statistical tests appropriate for the study design. One simple indicator of non-linearity is that variance is proportional to the mean. Data that fail these tests can usually be normalized by a log transformation so that all statistical analyses are carried out on transformed data. Means should be re-transformed to their original units to facilitate the interpretation of the results. An alternative is non-parametric statistical analysis.

The appropriate analysis for comparing responses among sites where sex or some other variable may also have an effect, and where sample sizes are often unequal, is analysis of variance (ANOVA) following the general linear model. The sex effect on MFO activity is seasonal. If it is non-significant during the inter-spawning interval, it may be omitted from the model to give more power to the ANOVA.

Where ANOVAs have been applied, differences among sites should be tested with an appropriate multiple range test such as Tukey's Test. A more sensitive alternative is to apply a priori contrast tests (Hoke *et al.* 1990). These are fixed comparisons (e.g. control vs test sites) built into the ANOVAs to test specific hypotheses about increased MFO activity downstream of a suspected source of inducers. All differences and effects should be tested at a 95% probability level. If preliminary estimates of variance are available, the minimum sample size to detect a

given difference (usually doubling of enzyme activity) and the probability of failing to detect a real difference (Type II error) should be calculated in advance as an aid to the study design. Where activity is significantly elevated above reference levels, the degree of induction must be calculated as the ratio of mean activities at treated sites to that at the reference site.

4.4 Definition of a response

There are no absolute or 'correct' values for MFO activity of fish. The AHH and EROD activities of white sucker from reference sites are usually less than 1.5 $FU.mg^{-1}$ protein in a 20 minute incubation for AHH and less than 10 $pmol.mg\ protein^{-1}.min^{-1}$ for EROD in the S-9 fraction or 30 $pmol.mg\ protein^{-1}.min^{-1}$ for the microsomal fraction. In contrast, these values may encompass the activities of induced fish of other species. Therefore, the best basis for judging the influence of an effluent is not the absolute activity, but the pattern of responses among contaminated and reference sites. Assuming that sufficient fish have been captured and analyzed at a minimum of one reference and two or more contaminated sites, an MFO response will be significant if criterion (a) and either criteria (b) or (c) are met:

a. Activity is significantly higher at contaminated sites relative to reference sites ($p<0.05$). Induction of activity by 10 to 40-fold is not uncommon when contaminated sites are compared to reference sites.

b. There is a decrease in activity with distance from the source of the effluent, suggesting an 'exposure-response' relationship due to dilution or transformation of the inducer. A difference could be seen within a short distance if there is dilution by a major tributary or if the effluent enters an open lake, estuary or marine ecosystem (e.g. Munkittrick *et al.* 1991a). In a river with no major tributaries, the minimum distance downstream between stations should be about 30 km or below a major obstruction to fish migration (e.g. Hodson *et al.* 1991). For shorter distances, the gradient may be so shallow that it is difficult to detect.

c. The MFO response is consistent between sexes of the same species, consistent among two or more species, or consistent among repeated surveys.

4.5 Presentation

All raw data must be presented along with means, standard deviations, sample sizes, and results of all statistical tests of comparisons among sites and correlations among variables. Where reference samples have been analyzed as an internal standard, the mean and standard deviation of the current results must be presented and compared with previous assays. Presentations of means and standard deviations in bar charts, showing reference and contaminated sites, are very helpful in understanding the results. The data must also be summarized in writing and any

difficulties with sample collection and analysis must be described and interpreted, as well as any unusual observations or results. Where other measurements have been made (e.g. temperature or chemical concentrations in sediments), it is important to compare them with the fish measurements as a way of explaining or eliminating possible causes of observed responses.

5.0 QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

Most of the difficulties in interpretation are associated with verifying that a negative response equals 'no induction', and not poor enzyme assays. There are several aspects to QA/QC which must be incorporated into every report. These include the results of assays of blanks, replicates, and standards, and the repeatability of the assay.

5.1 Blanks and replicates

Blanks are simply reaction mixtures without enzyme activity. Activity may be prevented by omitting an essential component such as NADPH or tissue extract, or by inhibiting the enzyme with a solvent such as methanol or acetone; appropriate baln s are described in each protocol. Blank values are normally very consistent but both blanks and tests must be run in triplicate when there are fewer than 24 samples. For larger numbers of samples, the numbers per sample may be reduced.

There have been several problems reported with blanks. If disposable tubes are used for fluorometric assays, there are differences in background fluorescence among and within batches of tubes. Some types and brands of disposable tubes show some natural fluorescence. If the laboratory is using reusable tubes, there may be persistent contamination and high blank values. Tubes must be carefully checked for consistency and low background fluorescence before each batch of samples is assayed.

5.2 Standards

There are two types of standards to run with most catalytic assays: internal and external (positive) controls. For internal controls, there is a significant amount of variability in the quality of substrates such as ethoxyresorufin and resorufin among suppliers and batches. Careful consideration must be given to procedures for monitoring batch-to-batch variability. For example, stock solutions of 7-ethoxyresorufin must be made up to a consistent peak absorbance (i.e. dissolved in DMSO until absorbance at 461.5 nm = 1.6 to 1.7).

Instrument checks must be made by diluting standards used for the standard curve in methanol. One high and one low standard must be run with each batch of samples to check instrument response. For example, in the spectrofluorometric EROD assay (Appendix B) these may be 0.0005 and 0.02 mg mL^{-1} resorufin standards diluted 10 µl into 2.5 mL of methanol.

It is essential that a laboratory reporting a negative response (no induction) assures that the assay was capable of detecting induction, if it was present. There are several ways to confirm or validate negative responses:

i) External positive controls to show induction was measurable:

a) Some laboratories induce MFO activity in fish by exposing a number of laboratory rainbow trout to an intraperitoneal injection of 0.5 $mg.kg^{-1}$ ß-naphthoflavone for a 2 - 5 d period. Livers are collected, homogenized, pooled and centrifuged to yield a large batch of homogenous microsomes. The microsomes may be divided into small aliquots and stored at -80°C for extended periods of time. A sample is thawed and run with each test batch to ensure consistency of measurements. Livers from fish sampled at reference and contaminated sites can be used in the same way.

b) It is possible to buy liver S-9 (such as rat liver preparations commonly used for Ames testing) and to use this as a consistent, positive, external control. This method has not been validated.

ii) Measurement of the P-450 protein:

For samples showing no induction, at least one sample from the contaminated site should be analyzed for P-450 content to ensure that the enzyme has not degraded during storage or handling. During degradation, the P-450 enzymes are reduced to P-420 forms, detectable through spectrophotometric analyses (Appendix E).

iii) Background data:

All assays must report protein levels, levels of reagents and incubation time to ensure that quenching or loss of substrate did not play a role in depressing activity.

iv) Protein:

Quality control standards are available commercially and must be included with each batch of protein assays.

5.3 Equipment

A variety of equipment is available for all aspects of MFO studies. For fluorometry, instruments range from simple filter fluorometers with relatively wide band widths to sophisticated spectrofluorometers with monochromators, narrow band widths, and continuously adjustable excitation and emission wavelengths. A comparison of several machines illustrated that, for the spectrofluorometric EROD assay, there were few differences among a Turner 112 filter fluorometer, Rayonics-Nova spectrofluorometer and Perkin Elmer spectrofluorometer in standard curves for resorufin and ethoxyresorufin (B. Kirner and P. Martel, Pulp and Paper Research Institute of Canada, 570 St. John's Boulevard, Pointe Claire, Québec H9R 3J9, personal

communication). Results of EROD assays on fish livers were virtually identical, except when staining of the flow cell on the Perkin Elmer machine caused elevated readings. This was eliminated through methanol rinses between samples. To ensure that equipment used for MFO assays gives high quality results, laboratories should periodically participate in inter-laboratory exchanges of samples and must use quality control standards as described in Section 5.2.

6.0 SUMMARY

This document has described several aspects of the application of MFO assays to monitoring the environmental effects of BKME. A number of options have been presented as well as factors that can affect the quality of data and the conclusions that may be drawn from results. It is obvious that the number and complexity of tests and study designs is large and that those who apply these techniques in biomonitoring programs will have to make some choices, depending upon their specific goals and circumstances. As a minimum, any monitoring program must have test and reference sites, some measure of MFO activity in a reasonable number of fish, and measures of a few associated variables to ensure that major biases have been accounted for. This minimum program is outlined in a flow diagram of a typical study (Figure 1), and it identifies where in this document supporting information can be found. The minimum measurements described in this protocol are also listed in Table 2. With this minimum program, the spatial variation of MFO activity can be described and the following questions answered:

- Is the MFO activity of fish at contaminated sites induced relative to that of fish at reference sites?
- Does the pattern of induction suggest that the source of inducers is the pulp mill?
- Does the intensity of induction suggest the need for more chemical and biological studies of contamination and effects on fish?

As indicated in earlier sections, additional numbers and types of measurements will aid the understanding of both the nature and causes of the observed changes.

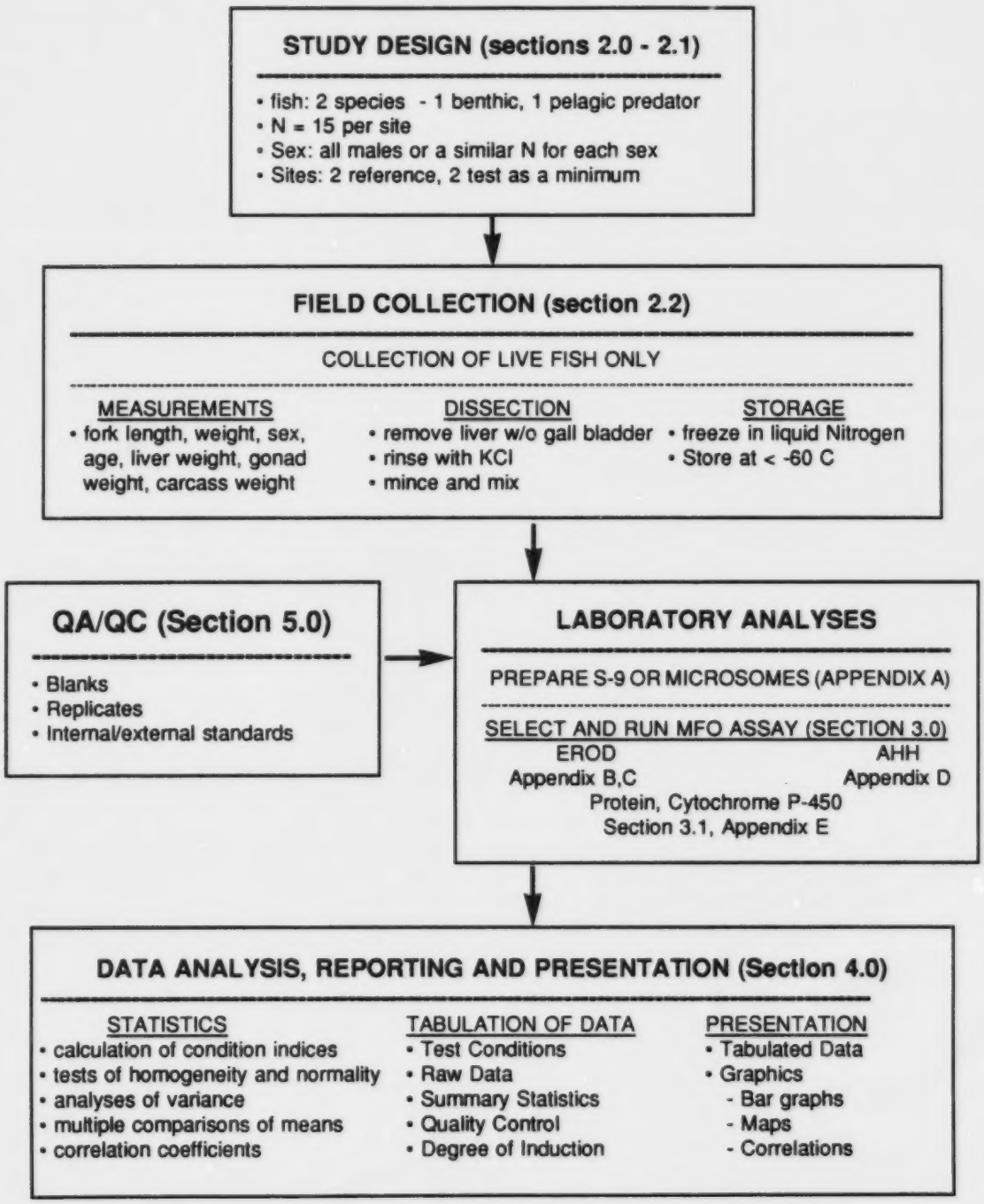
STUDY DESIGN (sections 2.0 - 2.1)
• fish: 2 species - 1 benthic, 1 pelagic predator
• N = 15 per site
• Sex: all males or a similar N for each sex
• Sites: 2 reference, 2 test as a minimum
FIELD COLLECTION (section 2.2)
COLLECTION OF LIVE FISH ONLY
MEASUREMENTS
• fork length, weight, sex, age, liver weight, gonad weight, carcass weight
DISSECTION
• remove liver w/o gall bladder
• rinse with KCl
• mince and mix
STORAGE
• freeze in liquid Nitrogen
• Store at < -60 C
QA/QC (Section 5.0)
• Blanks
• Replicates
• Internal/external standards
LABORATORY ANALYSES
PREPARE S-9 OR MICROSOMES (APPENDIX A)
SELECT AND RUN MFO ASSAY (SECTION 3.0)
EROD
Appendix B,C
AHH
Appendix D
Protein, Cytochrome P-450
Section 3.1, Appendix E
DATA ANALYSIS, REPORTING AND PRESENTATION (Section 4.0)
STATISTICS
• calculation of condition indices
• tests of homogeneity and normality
• analyses of variance
• multiple comparisons of means
• correlation coefficients
TABULATION OF DATA
• Test Conditions
• Raw Data
• Summary Statistics
• Quality Control
• Degree of Induction
PRESENTATION
• Tabulated Data
• Graphics
- Bar graphs
- Maps
- Correlations

Figure 1. Components of a study of MFO induction in fish

Table 2. Measurements required as part of a survey of fish liver MFO activities.

ACTIVITY	MEASUREMENT	MINIMUM NUMBER PER SITE AND SPECIES	PRECISION
WATER ANALYSIS	Temperature	1 depth profile	1°C
	pH	1, surface	0.1 units
	conductivity	1, surface	2% of working range
FISH SAMPLING	Total Weight	15	1 g
	Gutted weight (carcass)	15	1 g
	Fork Length	15	0.1 cm
	Sex	15[1]	-
	Liver weight	15	0.1 g
	Gonad weight	15	0.1 g
	Age	15	1 year
	Condition Factor	15	calculated
	Gonad Somatic Index	15	calculated
	Liver Somatic Index	15	calculated
ENZYME ANALYSIS (EROD)	Ethoxyresorufin - absorbance of stock solution	once	1.0 fluorescent units
	Resorufin standard curve	once, 5 points	'r' for standard curve[2] regression > 0.90
	Fluorescence of tests	15 in triplicate	1.0 fluorescent unit
	Protein standard curve	once, 5 points	'r' for standard curve[2] regression > 0.90
	Protein in test solutions	15 in triplicate	0.005 abs. units
	Calculated activity	15	0.5 pmoles resorufin. $.min^{-1}.mg\ protein^{-1}$
	Blanks	15 in triplicate	
	Activity of 'Standard' microsomes[3]	2 in triplicate	0.5 pmoles resorufin $.min^{-1}.mg\ protein^{-1}$

[1] Since sex can affect the activity of MFO enzymes, there should be roughly the same number of each sex at each site.

[2] 'r' is the correlation coeffcient for the regression of fluorescence on concentration.

[3] 'Standard' microsomes are those prepared in bulk from a large volume of liver and deep-frozen in aliquots for repeated analysis.

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9.0 APPENDICES - DETAILED METHODS

As indicated in Section 3.0, there are many methods and variations on methods for measuring MFO activities. The methods in these Appendices are those used by one or more of the authors of this report, and are presented because they have proved useful in field studies of fish MFO activity.

APPENDIX A. Preparation of S-9 fractions and microsomes

All subsequent methods test extracts of whole liver. The S-9 fraction is the supernatant generated by centrifuging a liver homogenate at 9,000 x g. Microsomes are precipitated by a 100,000 x g centrifugaton of the S-9 fraction.

A.1 Reagents and solutions

a) Potassium chloride - HEPES buffer (0.15 M KCl; 0.02 M HEPES; pH 7.5) (HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid)
b) Glycerol buffer - 0.05 M tris-(hydroxymethyl)methylamine (tris), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, and 20 % glycerol; pH 7.4.

A.2 Equipment

a) refrigerated ultracentrifuge and appropriate rotor
b) refrigerated superspeed centrifuge and appropriate rotors
c) motorized glass-teflon tissue homogenizer (Potter-Elvejhem type)
d) balance
e) tissue mincer (scissors or chopper of some sort)

A.3 Precautions

The steps described in this procedure must be carried out in a cold room at 1 - 4°C. Alternatively, all reagents, equipment and samples should be held on ice. Do not warm up the tissues by hand contact or by friction during homogenization. If subsamples of frozen specimens are desired, remove a portion while the specimen is still frozen and immediately return the unused part to the freezer. Under no circumstances should the sample be allowed to thaw and refreeze. Microsomes which are resuspended in KCl-HEPES must be kept in a refrigerator or immersed in ice until use.

A.4 Procedure

1) Subsample 1 - 2 g of frozen liver and transfer to a pre-weighed and pre-chilled petri dish. Immediately return the unused part of the liver to the freezer.

2) Weigh the liver and dish and calculate the weight of liver.

3) Allow the tissue to thaw or semithaw in a cold room at 1 - 4°C or on ice; the tissue must be soft enough to be cut up.

4) Mince the tissue with scissors or some other device and transfer to a prechilled tissue homogenizer. Conical tipped homogenizers of about 15 mL capacity work well.

> Note: For tissues minced and frozen in the field at the time of capture, pre-weigh an ice-cold homogenizing tube and transfer a semi-thawed portion of the sample to the tube for weighing.

5) Add 4 mL of cold KCl-HEPES per gram of tissue and homogenize with 5 - 7 passes of a motor driven teflon pestle (Potter-Elvejhem homogenizer)

6) Transfer the homogenate to polycarbonate tubes and centrifuge at 9000 x g for 30 min at 2 - 4°C. The post-mitochondrial supernatant (S-9 fraction) produced in this step may be analyzed directly if microsomes are not required. The supernatant is collected with a Pasteur or Eppendorf pipet, taking care to avoid the pellet and the floating lipid layer.

7) Transfer the cold supernatant to ultracentrifuge tubes. Keep them ice-cold while filling and balancing.

8) Centrifuge at 100,000 x g for 75 min at 2 - 4°C.

9) Remove the supernatant and wash the pellet 3 times with cold KCl-HEPES.

10) Using a glass-teflon tissue homogenizer, resuspend the pellet in sufficient cold KCl-HEPES to give a final protein concentration of 5 - 15 mg mL^{-1}.

11) Store the suspension in an ice bath or fridge and proceed to the enzyme assay. Assays should be completed within several hours. Frozen storage of microsomes resuspended in KCl-HEPES is not recommended. If microsomes are to be frozen they should be washed and resuspended in glycerol buffer. Use liquid nitrogen for freezing the microsomes.

> Note: Samples lose less activity during long-term storage (months) if frozen as a microsomal preparation than when frozen as whole tissue.

APPENDIX B. Ethoxyresorufin-o-deethylase (EROD) - spectrofluorometric analysis

This procedure is based on the method of Pohl and Fouts (1980), and has been the most commonly used in studies of BKME. There are two approaches to the estimation of EROD activity. The first is to follow the reaction as it occurs in the cuvette of a spectrofluorometer, measuring the appearance of resorufin, the product of the reaction (Prough *et al.* 1978). This technique requires a temperature-controlled cuvette and a recording device such as a chart recorder or computer graphics software. The advantages of this technique are those of simplicity and the ability to recognize immediately if the reaction is non-linear due to substrate or co-factor depletion. This method is not currently used by the authors but is a very suitable alternative. It is described in detail by Addison and Payne (1986).

The alternate technique, used when temperature control of the sample cuvette is not possible, requires a reaction in a water bath for a fixed and accurate time (Pohl and Fouts 1980). Methanol is used to stop the reaction and causes the denaturation and precipitation of proteins, so that separation of protein from the reaction solution is necessary before spectrofluorometry to prevent turbidity and interference. The first approach is obviously the simplest and is preferable, but may be limited by the availability of equipment. Both techniques give equivalent results, and the fixed-time method has been most frequently used in studies of pulp mill effluents.

B.1 Reagents and solutions

a) HEPES buffer (pH 7.8, 0.1 M) (HEPES = N-2-Hydroxyethylpiperazine -N'-2-ethanesulphonic acid)
b) magnesium sulphate (154 $mg.mL^{-1}$)
c) NADPH (0.5 mM or 27.5 $mg.L^{-1}$) (NADP = nicotinamide adenine dinucleotide phosphate; the 'H' refers to the reduced form)
d) 7-ethoxyresorufin (0.03 - 0.06 $mg.mL^{-1}$ in dimethyl sulphoxide) - see notes
e) BSA (bovine serum albumin) (40 $mg.mL^{-1}$)
f) Resorufin (see discussion on standards)
g) S-9 or microsomal preparation, as described in Appendix A

Notes:

a) Reagents a, b, and d may be prepared in bulk and stored in a refrigerator or cold room.
b) NADPH solutions should be prepared fresh immediately before use. The working time is 30 minutes.
c) There have been some problems with the concentration of ethoxyresorufin from different preparations of the purified compound. Klotz *et al.* (1984) describe methods for checking the purity of the ethoxyresorufin. Analysts should be aware of possible purity problems and take appropriate precautions.
d) 7-ethoxyresorufin is stored in a light-proof bottle at room temperature.

e) Since NADPH is very unstable, with a working life in solution of about one-half hour, an alternative is to use a biochemical system for generating NADPH. This requires the addition of 10 µL of each of the following solutions in place of the 30 µL of NADPH:
   f) NADP (98.4 mg mL^{-1})
   g) sodium isocitrate (193.58 mg mL^{-1})
   h) isocitrate dehydrogenase (usually purchased in solution and used as supplied - store in the refrigerator)

B.2 Instrumentation

a) fluorometer (spectrofluorometer recommended)
b) recording device for fluorometer output
c) Eppendorf pipettes (repeaters and adjustable)
d) vortex mixer
e) temperature-controlled water bath
f) high speed centrifuge and appropriate rotor (centrifugation to 100,000 x g)
g) stopwatch

B.3 Standards

Aliquots of 10 µl of resorufin standards (0.0005 to 0.02 $mg.mL^{-1}$ in dimethyl sulphoxide) are spiked into killed reaction mixtures (described below) and the fluorescence determined along with the fluorescence of a reaction mixture blank. Standard fluorescence is corrected for blank fluorescence and a response factor ($nmol.FU^{-1}$) in the reaction mixture (3.75 mL) is calculated and used in determining the amount of resorufin present in the unknowns. The preparation of standards is critical since the fluorescence produced is quite sensitive to the proportions of alcohol and water in the mixture being measured. Re-standardization may be required if instrument sensitivity changes or changes in reagents are made which affect the response.

These stock standards are stored in light-proof bottles at room temperature. Stability should be checked periodically if they are to be re-used. Klotz *et al.* (1984) discuss procedures for checking the purity of the resorufin preparation.

B.4 Procedure - fixed time assay

Three replicate assays and three blanks are recommended for each sample (6 tubes per sample). Procedures are the same for the assay tubes and the blank tubes up to step 5. Also, protect ethoxyresorufin and resorufin from exposure to strong laboratory lighting.

1) Into corex glass centrifuge tubes pipet the following reagents:
   - 1100 µl of HEPES buffer
   - 10 µl of $MgSO_4$
   - 50 µl BSA
   - 30 µl of NADPH

Note: The alternative method of generating NADPH from an enzyme system requires the addition of 10 µl of NADP, 10 µl of sodium isocitrate and 10 µl of isocitrate dehydrogenase (1.0 unit) in place of the 30 µl of NADPH. If the volume of isocitrate dehydrogenase required for one unit is greater than 10 µl, the volume necessary for one unit (to the nearest 10 µl) should be added and the volume of buffer reduced accordingly. The incubation mixture volume should be kept constant for comparability with the standards. Mix on a vortex mixer and allow the samples to stand for at least 10 minutes to ensure that sufficient NADPH is produced for the EROD reaction.

3) Add 50 µl of the S-9 or microsome preparation to each tube.

4) Incubate the tubes for at least 5 min in a water bath at an appropriate temperature (25°C for fish).

The following 2 steps (5, 6) apply to actual assay tubes only (not blanks).

5) Add 10 µl of ethoxyresorufin substrate to each tube and incubate for exactly 2 minutes in the water bath at the appropriate temperature. Use a stopwatch for timing.

6) After incubation, kill the reaction by adding 2.50 mL of methanol to each reaction tube and mix well. For very low activity samples, the incubation time may be increased by several minutes to allow for more conversion of substrate. Conversely, for very high activity samples, care should be taken that serious substrate depletion does not occur.

7) To prepare the blanks, add 2.50 mL of methanol to each tube to destroy the enzyme activity. Add 10 µl of ethoxyresorufin substrate solution. Precise timing is not required for these additions.

8) Centrifuge samples at 23,000 - 25,000 x g for 5 min to pellet the precipitated protein.

Note: When EROD reactions are stopped by adding methanol to the reaction mixture, proteins are precipitated and must be removed. This requires either a short centrifugation or a filtration through 0.8 µm glass fibre filters in a multi-port vacuum filtration system (e.g. Burdick and Jackson 12 or 24-port filtration unit, Canlab). A 24-port system allows filtration of up to 80 samples per hour. The advantages of filtration over centrifugation are those of speed and of low contamination. Pipetting the supernatant from centrifuge tubes risks contamination from the precipitated protein, whereas filtrates can be collected automatically, ready for fluorometry.

9) Transfer the clear supernatant to the fluorometer cuvette, taking care not to transfer any particulate matter, and measure the fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. Record the responses.

10) The protein content of the tissue preparation must be determined by accepted methods such as the Lowry, Biuret or BIORAD procedures.

11) Calculate the enzyme activity using the formula below.

activity = (f x pk)/((p x 0.050) x t)

where:

activity = enzyme activity ($nmol.min^{-1}.mg^{-1}$)

f = instrument response factor in n $mol.FU^{-1}$ for the reaction volume of 3.75 mL as determined from the standards.

pk = sample fluorescence corrected for blank fluorescence (FU).

p = protein concentration of the tissue preparation in $mg.mL^{-1}$.

t = incubation time in min.

B.5 Discussion

Activity increases linearly with protein concentrations up to 1 $mg.mL^{-1}$ in the reaction mixture. Care must be taken if the enzyme activity is very high to ensure that serious substrate depletion does not occur over the course of the assay. This may occur if high concentrations of very active protein are used.

The sensitivity of the method will depend on the instrumentation used. The absolute sensitivity is about 0.5 pmol of resorufin in the reaction mixture when using a spectrofluorometer.

APPENDIX C. Ethoxyresorufin-o-deethylase - spectrophotometric analysis

Ethoxyresorufin (7-ER) is an artificial substrate whose de-ethylation is catalyzed by a specific, inducible cytochrome P-450 (P-450IA). Original methods described a direct fluorometric technique for the measurement of the product (Burke and Mayer 1974). In the spectrophotometric assay, the generation of the product resorufin is monitored continuously at 572 nm (Klotz *et al.* 1984). Resorufin formation is dependent on the presence of enzyme, substrate and co-factor (NADPH). Temperature control is necessary during both pre-incubation and analysis.

In the spectrophotometric EROD assay, the formation of the product resorufin (pink) due to cleavage of the substrate (orange) by P-450IA is followed spectrally at 572 nm. The assay is a modification of the original fluorometric EROD assay. This assay has advantages over other methods in terms of substrate type since ethoxyresorufin is not a known carcinogen, as is benzo(a)pyrene. It uses a visible spectrophotometer rather than a fluorometer and the assay is easier. Interpretation is simpler - as a continuous assay, linearity is known and replicates can be added as needed immediately, rather than as later repetitions necessary in batch incubation assays. In addition, the daily blanks and standard curves needed for fluorometric and radiometric methods are not necessary with this procedure. However, some optimization may be necessary to gain the desired sensitivity for the very low activity expected in some cold water, unexposed fish. Although daily blanks are not needed for the spectrophotometric EROD assay, they should be run occasionally (e.g. all components minus NADPH, or minus enzyme). This is especially important if the assay indicates activity (increase in A_{572}), but no pink colour is formed, even after standing overnight. If pink colour (resorufin) is not present, it is likely that the change in A_{572} is NADPH and/or substrate independent, and overestimates true EROD activity. The original paper describing the method (Klotz *et al.* 1984) should be read by anyone setting up these assays. This protocol follows the Klotz method and describes some aspects not included in that reference.

C.1 Reagents and solutions

a) Ethoxyresorufin (Molecular Probes Inc. Eugene, Oregon)
b) HPLC-grade Methanol
c) NADPH (Sigma): 25 $mg.mL^{-1}$ in resuspension buffer. Make fresh daily or thaw a frozen aliquot
d) EROD Buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl)
e) Resuspension Buffer (50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 20% glycerol v/v)
f) S-9 or microsomal preparation, as described in Appendix A.

C.2 Equipment

a) Recording Visible Spectrophotometer (e.g. Shimadzu, Beckman, Perkin-Elmer)
b) Glass cuvettes (1 cm)

c) Water Bath. Circulating water baths can also be used with thermally-regulated cell holders in most spectrophotometers, to obtain temperature controlled preincubation as well as temperature-controlled cuvettes. Otherwise, a standing or shaking water bath can be used, if the spectrophotometer has its own built-in temperature control.

C.3 Preparation of 7-ER/EROD solution and sample 7-ER calculation

Calculate the amount of substrate needed as follows:

In a total assay volume of 500 µl, there will be:

a) 10 µL of microsomes (add more or dilute sample as needed, see Appendix C.4, Step 5 below)
b) 10 µL of 25 $mg.mL^{-1}$ NADPH (in resuspension buffer), and
c) 480 µL of 7-ER in EROD buffer

A saturated solution of 7-ER in methanol has an approximate concentration of 400 µM. A final concentration of 2 µM is needed. Thus, 2/400 or 0.005 times the total volume is desired. For 480 µL per run, this is 2.4 µL per run.

If 12 samples are to be assayed, assume 3 replicates (12 x 3 = 36) and add a few spares to make enough 7-ER mixture for 45 assays. For 45 samples:

NADPH:	45 assays x 10 µL/assay	= 450 µL at 25 $mg.mL^{-1}$
7-ER:	45 assays x 2.4 µL/assay	= 108 µL 7-ER
EROD buffer:	45 assays x 480 µL/assay	= 21.6 mL EROD buffer

Therefore, add 108 µl of 7-ER to 21.6 mL EROD buffer to approach the target of 2 µM. Zero the spectrophotometer at 482 nm with EROD buffer alone in both the reference and sample cuvettes. Then measure the Absorbance at 482 nm of the 7-ER/EROD buffer mixture versus the "zeroed" straight EROD buffer. The extinction coefficient of 7-ER is 22.5 $mM^{-1}.cm^{-1}$, and the 7-ER/EROD mixture will comprise only 480 of the final volume of 500 µL. Thus, the actual final concentration of 7-ER in the assay is calculated as:

Concentration of 7-ER (µM) = $(A_{482}/0.0225)$ x (480/500)

If this number is 2.0 $\pm$ 0.1, use it. BUT:

1) If the 7-ER concentration is too low, use this correction:

New total volume of 7-ER = (2/(calculated uM)) x (original 7-ER volume)

Subtract the original volume of 7-ER used and add this amount. Re-measure the new substrate, mix, and reiterate as necessary.

Example: A_{482} = 0.040, then

Concentration of 7-ER	= (0.040/0.0225) x (480/500) = 1.70 uM	
New total volume of 7-ER	= (2/1.7) x 108	= 126.5 µL
Amount of 7-ER to add	= (126.5 - 108)	= 18.5 µL

2) If the 7-ER concentration is too high, use a similar correction, but now for buffer volume:

New buffer volume = ((Calculated uM)/2 uM) x (original buffer volume)

Subtract the original buffer volume and add this amount. Remeasure the new substrate mix and reiterate as needed.

Example: A_{482} = 0.053, then

Concentration of 7 -ER	= (0.053/0.0225) x (480/500) = 2.26 µM	
New Buffer volume	= (2.26/2) x 21.6	= 24.4 mL
EROD buffer to add	= (24.4 - 21.6)	= 2.8 mL

It may take some adding of both or either component(s) to achieve the correct concentration of 7-ER in EROD buffer, but this becomes easier with time as the operator learns what the desired solution looks like.

C.4 Procedure

1) Set the water bath for preincubation to the optimum temperature determined for the test species, but keep the spectrophotometer at room temperature until the substrate is prepared and read at 482 nm. Prepare samples for analysis according to the method for microsome or S-9 preparations (Section 7).

2) Make substrate: dissolve a crystal of 7-ethoxyresorufin (7-ER) in 0.5 mL HPLC-grade MeOH. Place the test tube in hot water to aid dissolution. Let the solution cool to room temperature. 7-ER dissolved in MeOH will degrade with time, so a fresh sample must be prepared daily. Calculate the number of samples to be run and make a sufficient volume of 7-ER in EROD buffer. Check the concentration of 7-ER at 482 nm and adjust as needed to a final concentration of 2 µM (see example under "Preparation of 7-ER/EROD Solution" (Appendix C.3)). Keep the 7-ER/EROD buffer mixture on ice until used.

3) Change the wavelength from 482 nm (for the 7-ER reading) to 572 nm to measure the resorufin formation. Prepare the spectrophotometer for time-course parameters, and bring the cuvettes to a proper temperature. Circulate water if a water bath is used to

control cuvettes, or set the cuvette holders to the same temperature as the preincubation bath if a separate system is used. Pipette 480 µL 7-ER/EROD mixture into a few small test tubes, to bring the substrate to room temperature and to reduce the time lag for bringing it to the assay temperature.

4) Add microsomes (try 10 µL first) to 480 µL 7-ER/EROD solution and pre-incubate in the water bath for 2-3 minutes.

5) Remove the tube from the bath and quickly add 10 µL NADPH to initiate the reaction. Transfer the solution to the sample cuvette and record the change in absorbance at 572 nm against EROD buffer in the reference cuvette. Because this is a kinetic assay, temperature control is important. The rate of resorufin formation should be linear for at least 3 min. Bubbles or settling may initially obscure the observable rate of change in absorbance at 572 nm. It is not necessary to always begin recording the change in absorbance at the same interval after NADPH addition, but note that the enzyme is not stable in EROD buffer and will start to degrade over time, losing activity. Thus, extremely long pre-incubations and run times should be avoided.

   If the change in absorbance is too fast or too slow, repeat the assay with less or more sample. Dilute microsomes in resuspension buffer or use more microsomal volume, respectively.

   Note: If the total assay volume is changed significantly, recalculate the 7-ER concentration to assure it is still 2.0 ± 0.1 µM.

6) Run replicates of each sample. To save time, pre-incubate the next sample in the water bath while first one is being monitored.

7) Using the linear change in absorbance, calculate the EROD activity. This calculation is based on the observed change in absorbance per min at 572 nm, the extinction coefficient of resorufin at 572 nm (73 $mM^{-1}.cm^{-1}$, Klotz *et al.* 1984), a correction factor, and the total assay volume and volume of enzyme added. A dilution factor is needed if samples have been diluted for the run. To obtain an activity per unit volume, calculate the following:

   nmol resorufin formed.$min^{-1}.ml^{-1}$ = (delta A_{572}/min) x (mmol/73 L) x (10^6 nmol/mmol) x (1/1000 mL) x (500/10) x dilution)

8) To determine the EROD specific activity (nmol.min^{-1}.mg $protein^{-1}$), divide by mg.mL^{-1} protein.

9) To calculate the EROD turnover number (nmol resorufin.min^{-1}.$nmol^{-1}$ P-450), divide the specific activity by the P-450 specific content (nmol.mg^{-1}), or divide the original value (nmol.min^{-1}.mL^{-1}) by the P-450 content per volume (nmol P-450.L^{-1}). P-450 content is determined by spectral analysis as described in Appendix E.

Example:

Total Volume =	480 µL (7 ER + buffer)	+	10 µL (sample at 1:5 dilution)	+ 10 µL (NADPH)	=	500 µL,

If the measured delta $A_{572}.min^{-1}$ = 0.0108, then:

Activity = 0.0108 x 1000/73 x 500 µL/10 µL x 5 = 36.99 $nmol.min^{-1}.mL^{-1}$

If the protein concentration is 24.3 $mg.mL^{-1}$, then:

Activity = 36.99/24.3 = 1.52 $nmol.min^{-1}.mg^{-1}$ protein;

If the P-450 content is 0.37 $nmol.mg^{-1}$, then:

Activity = 1.52/0.37 = 4.11 $nmol.min^{-1}.nmol^{-1}$ P-450.

APPENDIX D. Aryl hydrocarbon hydroxylase (AHH)

Arylhydrocarbon hydroxylase (AHH) encompasses many enzyme systems, two of which use Benzo(a)pyrene (BaP) as a substrate or diphenyloxazole (PPO). There are published methods for BaP hydroxylase which use a radio-labelled BaP substrate (Nebert and Gelboin 1968a; 1968b; Binder and Stegeman 1980). The water-soluble product is extracted at the end of the assay and measured by standard scintillation counting. The advantage of this technique is that the product of the reaction is known and standards are available for quality control and for accurately estimating enzyme activity in terms of product generated. The major disadvantage of the technique is the use of a substrate that is highly carcinogenic and the use of solvent-based fluors for beta-counting. While this method may be quite suitable for surveys of BKME effects, details are not given because it is not currently used in the authors' laboratories due to its inherent hazards. A method is described by Addison and Payne (1989).

The PPO assay estimates the activity of aryl hydrocarbon hydroxylase (AHH) by measuring the appearance of a NaOH-extractable fluorescing metabolite of diphenyloxazole when incubated with enzymes extracted from liver tissue under standard conditions.

In comparing the PPO assay with the BaP hydroxylase assay there are a number of advantages and disadvantages. The AHH assay was modified from Nebert and Gelboin (1968a) using 2,5-diphenyloxazole (PPO) as a substrate because it is less carcinogenic than BaP. The PPO substrate is stable and the assay is faster and simpler than the BaP assay. The major drawback of the PPO assay is that the metabolism of PPO generates an unknown product; therefore, sample results must be compared in relative units only. The assay also produces lower AHH activities than BaP by about one-third because the S-9 supernatant is used instead of microsomes. Despite these simplifications in the method, the relative values of AHH activities from different sampling locations and from repeated sampling over several years at one site demonstrated significant and consistent trends in AHH activity (Hodson *et al.* 1989). Quality control in the PPO assay is derived by the use of known fluorescing compounds for calibrating the fluorometer and by repeated assays of livers with previously measured and known activities.

The EROD assay, by comparison, is safer than the PPO assay because solvents such as hexane are not used. The EROD sensitivity is also greater and specific activity in nmol product. $min^{-1}.mg^{-1}$ protein can be determined.

D.1 Reagents and solutions

a) PPO - (2,5-Diphenyloxazole): Treat this toxic chemical with respect. Wear personal protective equipment. Prepare PPO fresh for each AHH assay.

452 uM PPO - Dissolve 2 $mg.mL^{-1}$ using spectro-grade methanol.

To the PPO powder, add 1.0 mL of methanol at a time and carefully dissolve and transfer the solution into a labelled test tube. If more than 1.0 mL methanol is added to the weigh boat, it will be lost due to rapid evaporation. Vortex the solution. Add 50 µL PPO solution/AHH sample.

b) NADPH - 4 mM: Prepare fresh for each day's work. Dissolve 3.33 $mg.mL^{-1}$ of assay buffer. Transfer to a labelled test tube and then Vortex. Add 100 µL NADPH solution/ AHH sample (0.4 mM).
c) Assay Buffer: Dissolve 7.459 g of Tris buffer (pH 7.5) and 0.6095 g (3 mM) $MgCl_2$ in 1000 mL distilled water.
d) Quinine sulphate standard: Add 3.7335 mg Quinine sulphate to 100 mL of 0.1 N H_2SO_4
e) GC-grade hexane
f) S-9 preparation, as described in Appendix A.

D.2 Equipment

a) Shaking water bath with clips to hold 25 mL Erlenmeyer flasks
b) Turner fluorometer, Model 110, with emission filters 2A and 3, and excitation filter 7-60.
c) Vortex mixer
d) Micropipets

D.3 Procedure

1) Into three erlenmeyer flasks (25 mL, duplicate samples plus a blank) add:
   - 350 µL assay buffer
   - 100 µL NADPH
   - 500 µL liver supernatant

   Vortex the sample for every flask. The remaining supernatant is used for protein determination by the Biuret or Lowry method with bovine serum albumin (BSA) as a standard.

2) Ensure that the water bath is at 27°C. Place and secure flasks into the waterbath and restart the shaker.

3) Add 50 µL of PPO in methanol to the samples, leaving exactly 30 s between each flask. Time this action with a stopwatch. Incubate for 20 min.

4) Making sure there are no air bubbles in the repipettors, add to the blanks:
   - 1.0 mL acetone, swirl by hand
   - 3.25 mL hexane, vortex for 10 s
   - 50 µL of PPO, swirl by hand

   Set the blanks to one side for readings.

5) After the 20 min incubation, one has 30 s to process each flask one at a time, as follows:
   - add 1.0 mL acetone, swirl by hand for several seconds
   - add 3.25 mL hexane and vortex for 10 s

   Avoid spillage! Use a stopwatch to ensure that samples are processed in exact 30 s intervals.

6) Carefully decant the top hexane layer into a test tube.

7) Carefully transfer 2.0 mL of the sample into the next row of test tubes with an Eppendorf pipet. To prevent the hexane solution from dripping, pipet 1.0 mL and release it back into the original sample test tube. This will coat the pipet tip.

8) To the 2.0 mL samples, add 5.0 mL 1N NaOH and vortex for 30 s. Avoid spillage.

9) Allow the samples to settle into two phases.

10) Remove the bottom phase and transfer it to the last row of test tubes with a Pasteur pipet.

D.4 Fluorometer readings

1) Warm up a Turner 110 fluorometer for 30 min.

2) Standardize the fluorometer with emission filter No. 2A and excitation filter 7-60. Zero the machine with distilled water. Take a reading of the quinine sulphate standard. Readings must be consistent from day-to-day to ensure no drift.

3) Sample readings: Replace filter No. 2A with filter No. 3. Zero the machine with a sample blank. Read each sample. If a sample reading is greater than 100, dilute with 1N NaOH, ensuring that the total volume is 4.0 mL. Vortex the original sample when making the dilutions.

4) Calculate specific activity by dividing the reading of fluorescence units (FU) by the protein concentration, correcting for dilution if necessary. Specific activity is expressed as $FU.min^{-1}.mg\ protein^{-1}$. One FU is equivalent to a reading on a 1x scale of the fluorometer with quinine sulphate used to ensure no drift from day-to-day.

> Note: Alternate method: A spectrofluorometer may be substituted for a fluorometer, using an excitation wavelength of 345 nm and an emission wavelenth of 510 nm (Ahokas 1976).

APPENDIX E. Spectral P-450 analysis

The potential for protein denaturation exists for all samples, particularly for field samples which are shipped to laboratories for further analysis. Hence, the catalytic competence of representative samples must be proven occasionally by spectral scanning for total P-450 content. If the scan reveals a single peak at about 450 nm, the sample is intact and activity results are accurate (barring inhibition). If the scan reveals a significant peak at about 420 nm, the sample is degraded and activity is underestimated. If there is a peak present at 420 nm but not at 450, true activity will be absent. Cytochrome b5 can sometimes be induced by P-450 inducers, and its reductase activity may interact with other P-450 activities. If present, it can be observed during spectral analysis by following step 2 below.

Spectral analysis requires more sample volume than the spectrophotometric EROD assay and some training in practice and interpretation of results. However, if no activity or low activity is observed at all sites, this analysis is necessary. For convenience, 2 representative samples per site (e.g. with representative enzyme activities) should be analyzed for total spectral P-450 to determine enzyme competence.

E.1 Reagents and solutions

a) NADH (5 $mg.mL^{-1}$ in resuspension buffer)
b) CO (e.g. training bottle)
c) sodium dithionite crystals (Na_2SO_4)
d) resuspension buffer (see EROD spectrophotometric protocol)
e) S-9 or microsomal preparation, as described in Appendix A.

E.2 Equipment

a) scanning spectrophotometer, dual or single beam
b) glass cuvettes, 1 cm, with lids

E.3 Procedure

To determine TOTAL P-450 content, a reduced, CO-bound difference spectrum is measured. The following protocol is based on Stegeman *et al.* (1979). If cytochrome b5 values are desired, follow the method below. If cytochrome b5 is not needed, add 10 µl NADH to the diluted sample and skip step 2.

1) Dilute microsomes (or post-mitochondrial supernatant) in resuspension buffer to a final volume of about 0.7 to 0.8 mL for dual beam determinations, half that for single beam. Dilutions of microsomes prepared at a resuspension ratio of 1 mL $buffer.g^{-1}$ of liver should generally be diluted 1/20.

2) For dual beam determinations, divide the sample evenly between two cuvettes and record a baseline spectrum between 500 and 400 nm. Add 5 µL NADH to the sample cuvette and scan between 450 and 400 nm. Cytochrome b5 will show as a peak at about 424 and a trough at about 409 nm. This difference is used to determine the concentration of b5 using the extinction coefficient of 185 $mM^{-1}.cm^{-1}$ (see below). Combine the sample from both cuvettes and add an additional 5 µL NADH to reduce the b5 from the "reference" sample.

   For single beam determinations, run a baseline with the entire sample in the sample cuvette and store it, then run the NADH-reduced sample versus the stored baseline.

3) Bubble the sample with CO in a hood for about 30 s. Re-divide the sample between the reference and sample cuvettes and cover. Record a baseline spectrum at 500 to 400 nm as before. Some settling may occur; if so, wait and run the baseline again until stable. Add a few crystals of sodium dithionite to the sample cuvette and scan from 500 to 400 nm. As it may take 10-20 min for complete reduction, scan again until peaks are stable. Determine the P-450 concentration using 91 mM^{-1} cm^{-1} as the extinction coefficient, as shown below.

   For single beam determinations, establish a baseline with the CO-bubbled sample and store it, then run the dithionite-reduced sample against the saved CO bubbled baseline run.

4) Wash out the cuvettes, making sure all traces of dithionite are removed. Repeat the analysis with the next sample.

5) Calculations assuming a 1 cm path length:

   Cytochrome b5 ($nmol.mL^{-1}$) = $A_{(424-409)}$ x mmol/185 L x 10^6 nmol/mmol x (1 L/10^3 mL) x dilution

   Cytochrome P-450 ($nmol.mL^{-1}$) = $A_{(490-449)}$ x mmol/91 L x 10^6 nmol/mmol x (1 L/10^3 mL) x dilution

   Divide these results by the protein concentration ($mg.mL^{-1}$) to get specific contents, or $nmol.mg^{-1}$ protein.

   Note:

   a) Upwards skewing (towards the 400 nm end) of the P-450 spectrum often occurs if too much dithionite is added. Add only a few crystals, let the sample reduce, and add more if you suspect that not enough dithionite was added. Ideal P-450 scans will run flat from 500 to 470 nm before the peak starts to rise, and drop below zero by 400 nm. Calculations may be adjusted according to the scan obtained. In the calculation above, 490 is taken as the representative "zero"and 449 as the peak P-450 value. If the baseline is not flat, correct for this in calculating the P-450 value.

b) This analysis is usually run at room temperature. For most species, you can thaw a sample of microsomes, dilute it in resuspension buffer and let it sit on ice for a few hours until analysis. In fact, it may remain stable even after freezing overnight at -20 degrees; of course catalytic activity is reduced or eliminated. Stability should be tested for each species. For example, run one portion of a sample immediately after thawing and compare it to another portion left on ice for a few hours; let a fully-reduced sample sit in the cuvettes, scan over time, and observe whether a P-420 peak develops.

c) Dithionite will be "exhausted" quickly in a humid room. Store dithionite aliquots in a dessicator and use each aliquot only for one day. During humid conditions, change dithionite more frequently. Strong odour and/or "cling" indicate exhaustion of the chemical.

d) Remember to clear the CO lines of air by bubbling into water or buffer before CO bubbling the first sample of the day.

e) This assay measures TOTAL P-450, of which P-450IA may be a minor component. Therefore, changes in spectral hepatic P-450 are an inconsistent marker of exposure to inducing chemicals. This assay will give indirect information on P-450IA. For example, turnover numbers for catalytic activity will indicate whether the portion of P-450IA has increased. However, the most important function for spectral P-450 analysis is to check the overall functional capacity of the P-450 proteins. If there is more than a shoulder at 420, beware of catalytic results!